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Molecular cloning, differential expression, and functional characterization of a family of class I ubiquitin-conjugating enzyme (E2) genes in cotton (*Gossypium*)☆

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Abstract

Two cDNAs and their corresponding genes (*GhUBC1* and *GhUBC2*) encoding ubiquitin-conjugating enzymes (E2s) have been cloned and characterized from allotetraploid cotton *Gossypium hirsutum* ($(AD)_1$ genome). Three additional E2 genes (*GaUBC1*, *GtUBC2*, and *GrUBC2*) have also been identified from diploid cottons *Gossypium arboreum* (A_2 genome), *Gossypium thurberi* (D_1 genome), and *Gossypium raimondii* (D_5 genome), respectively. The derived amino acid sequences of the five closely related cotton E2s are 77–79% identical to yeast ScUBC4 and ScUBC5. The *GhUBC1/2* gene family is composed of two members, and genomic origin analysis indicates that *GhUBC1* and *2* are individually present in the A and D subgenomes of *G. hirsutum*. The transcript levels of *GhUBC1/2* increased significantly in leaves and flowers at senescence, suggesting that GhUBC1/2 may play a role in the degradation of target proteins that function in the delay of the senescence program. Correlated with high auxin content and auxin-associated effects, *GhUBC1/2* are also highly expressed in the youngest leaves, the apical part of lateral roots, and elongating fibers. Genetic complementation experiments revealed that GhUBC1 and 2 can substitute for the function of ScUBC4 and 5 required for the selective degradation of abnormal and short-lived proteins in a yeast *ubc4ubc5* double mutant.

Keywords: Gene expression; *Gossypium*; Senescence; Ubiquitin-conjugating enzyme; Yeast complementation

1. Introduction

The ubiquitin-proteasome pathway plays a central role in many cellular processes in eukaryotes. The pathway is responsible for the selective degradation of abnormal proteins and many short-lived regulatory proteins, including cell cycle proteins, signal transducers, cell growth modulators, and transcription factors [1]. The covalent attachment of ubiquitin to targeted proteins, a required step for the degradation of these substrates, is generally catalyzed by three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). Ubiquitin is first activated by E1 through the formation of a high-energy thioester bond between ubiquitin and E1. The activated ubiquitin is then transferred from E1 to a conserved cysteine residue of an E2. The E2 either by itself or often in combination with an E3 catalyzes the final attachment of ubiquitin to a target protein. The E3 can be a single protein or a multiple subunit complex. One of the best-characterized E3 complexes is the SCF complex that consists of four subunits: cullin, SKP1, RBX1, and an F-box protein [2]. The ubiquitinated protein is subsequently recognized and degraded by the 26S proteasome [3].

☆ The cDNA sequences of *GhUBC1* and *GhUBC2* as well as the nucleotide sequences of *GhUBC1*, *GhUBC2*, *GaUBC1*, *GtUBC2*, and *GrUBC2* genes have been deposited in GenBank under accession numbers AY082004, AY082005, AY082006, AY082007, AY082008, AY082009, and AY082010, respectively.

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Multiple E2 enzymes have been identified in yeast, plants, and animals. At least 10 different E2s (ScUBC1–10) have been characterized in the yeast *Saccharomyces cerevisiae* [4]. ScUBC4 and ScUBC5 are class I E2s, which contain only the conserved 16-kDa UBC domain and lack N- and C-terminal extensions. The two E2s are closely related in sequence and complementary in function and play a major role in the selective degradation of abnormal and short-lived proteins [5]. The expression of *ScUBC4* and *5* is induced by heat shock. The deletion of *ScUBC4* and *5* in the yeast *ubc4ubc5* mutant causes cell growth defects and inviability at elevated temperatures [5]. E2 homologs, which are highly similar to ScUBC4 and 5 at the amino acid sequence level, have been identified in diverse organisms, including human (*Homo sapiens*) HsUbcH5B and HsUbcH5C, fruit fly (*Drosophila melanogaster*) DmUbcD1, nematode (*Caenorhabditis elegans*) CeUBC-2, rat (*Rattus norvegicus*) RnUBC4-1 and RnUBC4-testis, fungus (*Colletotrichum gloeosporioides*) CgUBC1, *Arabidopsis thaliana* AtUBC8, 9, 10, 11, and 12, tomato (*Lycopersicon esculentum*) LeUBC, pea (*Pisum sativum*) PsUBC4, and rice (*Oryza sativa*) OsUBC [6–16]. AtUBC8–12 are generally expressed in root and leaf tissues. However, unlike yeast ScUBC4/5, the expression of these *Arabidopsis* class I E2s is not induced by heat stress [13]. The OsUBC protein, encoded by a single-copy gene, has been shown to be responsible for the derepression of α-amylase gene expression in rice seeds. Induced by gibberellin (GA), OsUBC is presumably involved in the degradation of a repressor-like protein that binds to the promoter region of the α-amylase gene in aleurone cells [16]. The transcript level of the tomato E2 gene, *LeUBC*, increased in senescent leaf tissues and developing fruits, and reached to a peak after the onset of fruit ripening [14]. The ScUBC4/5 homologs from animal and fungal species including *D. melanogaster*, *C. elegans*, and *C. gloeosporioides* can functionally complement proteolysis deficiency in a yeast *ubc4ubc5* double mutant [6–8]. However, so far it has not been shown that any ScUBC4/5 homologs from plant species can functionally substitute for ScUBC4 or 5 in yeast cells.

To better understand the physiological roles of ScUBC4/5-related E2s in cotton and other higher plants, we have begun to study the organization, expression, and function of the class I E2 genes in cotton. Here we report the cloning and characterization of five *ScUBC4/5*-related E2 genes from the allotetraploid and diploid cotton species: *GhUBC1* and *GhUBC2* from *G. hirsutum* ($(AD)_1$ genome), *GaUBC1* from *Gossypium arboreum* (A_2 genome), *GtUBC2* from *Gossypium thurberi* (D_1 genome), and *GrUBC2* from *Gossypium raimondii* (D_5 genome). We also present the results of transcript analysis of *GhUBC1* and *GhUBC2* in various cotton tissues and at different developmental stages. Furthermore, we demonstrate that GhUBC1 and GhUBC2 can complement the function of ScUBC4 and ScUBC5 in a yeast *ubc4ubc5* double mutant.

2. Materials and methods

2.1. Cloning and sequencing of class I E2 cDNAs

A cotton cDNA fragment (251 bp), *cUbc23*, encoding 1–27 amino acid residues of the N-terminal region of a ScUBC4/5-related E2, was ^{32}P-labeled and used as the hybridization probe to screen a cotton (*G. hirsutum* Stoneville 213) root cDNA library constructed in the λZAPII vector (Stratagene) [17]. Hybridization was performed in 5× SSPE, 50% formamide (w/v), 5× Denhardt's solution, 0.1% SDS (w/v), and 100 μg/ml denatured nonhomologous salmon sperm DNA at 42 °C for 12–16 h with constant agitation. After hybridization, membranes were washed twice in 2× SSC, 0.5% SDS (w/v) at room temperature for 10 min, twice in 1× SSC, 0.1% SDS (w/v) at 42 °C for 20 min, and finally in 1× SSC, 1% SDS (w/v) at 65 °C for 20 min. The positive λZAPII cDNA clones were excised in vivo with the VCS M13 helper phage to generate plasmid subclones in the pBluescript vector. The recombinant pBluescript DNAs were digested with *Eco*RI and *Xho*I, and the inserted cDNA fragments were then cloned into M13mp18 and M13mp19 vectors. The recombinant M13mp18 and M13mp19 single-stranded DNAs were prepared and sequenced using an ABI PRISM 310 DNA Genetic Analyzer (Perkin-Elmer).

2.2. Genomic southern analysis

Cotton genomic DNA was isolated as described by Paterson et al. [18]. Southern blot analysis was performed as described by Sambrook et al. [19]. The genomic DNA was individually digested with restriction enzymes *Dra*I, *Eco*RI, *Hin*cII, *Hin*dIII, and *Xba*I, electrophoresed on a 1% agarose gel, and transferred to Gene Screen Plus (Du Pont Inc.) nylon membrane with the alkaline transfer method. Hybridization of the membrane with the *cUbc23* probe was carried out in 5× SSC, 50% (w/v) formamide, 5× Denhardt's solution, 1% SDS, 10% dextran sulfate (MW 500,000), and 100 μg/ml denatured nonhomologous salmon sperm DNA at 42 °C for 12 h. After hybridization, the membrane was then washed twice in 2× SSC at 42 °C for 10 min, twice in 1× SSC, 1% SDS at 42 °C for 20 min, and then exposed to X-ray film with an intensifying screen at −70 °C for 3–5 days.

2.3. Determination of genomic sequences and origins of GhUBC1 and 2 genes

Two sets of primers, UBC-5-SL (5′-CAAGAATTCAAACTTTCCCGTCTCATATCTAATCC-3′) and UBC-3-S (5′-CGCAAGCTTAGGAAATGCCTTTCATA-3′), and

UBC-5-SL and UBC-3-L (5′-GCCAAGCTTACAACCA-TAGACCCACCTT-3′), designed from the *GhUBC1* and *GhUBC2* cDNA sequences, respectively, were used in PCR amplification to determine the genomic origins of *GhUBC1* and *GhUBC2* genes. One microgram of cotton genomic DNA isolated from young leaves of *G. arboreum* (A_1 genome, accession number A_1-57), *G. herbaceum* (A_2 genome, accession number A_2-86), *G. thurberi* (D_1 genome, accession number D_1-1), *G. raimondii* (D_5 genome, accession number D_5-4), and *G. hirsutum* St213 ($(AD)_1$ genome), respectively, was used as the template for the PCR reaction with REDTaq DNA polymerase (Sigma). The cotton genomic DNA was also amplified with *Pfu* DNA polymerase (Stratagene), a high fidelity enzyme, for the accurate sequence determination of the amplified cotton E2 genes.

2.4. Complementation of yeast ScUBC4/5 by cotton GhUBC1 and 2

S. cerevisiae wild-type *ubc4*, *ubc5*, *ubc4ubc5* mutants [5] were kindly provided by Dr. Stefan Jentsch. The PCR primers UBC-5-SL-EXP01 (5′-AGGAAGCTTGTGGT-CTGTTCAAAGCGTCAT-3′) and UBC-3-L-EXP01 (5′-CATGAATTCTTACAACCATAGACCCACCTT-3′), and UBC-5-SL-EXP01 and UBC-3-S-EXP01 (5′-CGC-GAATTCTAGGAAATGCCTTTCATA-3′) were used to amplify the cDNA sequences of *GhUBC1* and *GhUBC2*, respectively, with *Pfu* DNA polymerase. A *Hin*dIII (AAGCTT) or *Eco*RI (GAATTC) restriction site was engineered at the 5′ end of the primers. The amplified *GhUBC1* and *2* DNA fragments were digested with *Hin*dIII and *Eco*RI, and cloned into a pYES6/CT expression vector (Invitrogen) to generate two recombinant plasmids pYES6/CT-GhUBC1 and pYES6/CT-GhUBC2. The yeast *ubc4ubc5* double mutant was individually transformed with pYES6/CT-GhUBC1, pYES6/CT-GhUBC2, and pYES6/CT and then selected on YPG (yeast extract, peptone, and galactose) medium plates containing the antibiotic of blasticidin.

2.5. Northern blot analyses of GhUBC1/2 genes

Cotton (*Gossypium hirsutum* DES119) tissues including fibers, flowers, leaves, and roots at different developmental stages were collected and used in the isolation of total RNA with the method of Hughes and Galau [20]. Total RNAs (10 μg) were electrophoresed in a formaldehyde agarose gel and transferred to Gene Screen Plus membrane. The membrane was hybridized with the *GhUBC2* cDNA probe, which was amplified by PCR from a λZAPII/*GhUBC2* cDNA clone and labeled by [α-^{32}P] dCTP with the random priming method as described by Hodgson and Fisk [21]. After hybridization, the membrane was washed twice in 2× SSPE buffer at 42 °C for 10 min, twice in 1× SSPE, 1% SDS at 42 °C for 10 min, and exposed to X-ray film with an intensifying screen at −70 °C for 3–5 days.

3. Results

3.1. Cloning of GhUBC1 and GhUBC2 cDNAs encoding class I E2s from G. hirsutum

After screening about 1.8×10^5 recombinant phages of the λZAP II cDNA library with the labeled *cUbc23* probe, eight E2 clones were isolated and two unique cDNAs encoding ScUBC4/5-related E2s, named as GhUBC1 and GhUBC2 (Gh represents *G. hirsutum*) (Fig. 1), were identified by sequence analysis. Both *GhUBC1* and *GhUBC2* encode an E2 protein containing 148 amino acids. The two enzymes have a putative active cysteine (Cys) residue located at the amino acid position 85. The Cys residue is present in all E2 enzymes from different organisms and is required for the thioester bond formation with ubiquitin [22,23]. The *GhUBC1* and *GhUBC2* cDNA sequences share 80% identity at the nucleotide level (excluding two PolyA tail sequences and the extra 21-nucleotide sequence at the 5′ end of *GhUBC1*). High levels of identity are present in the open reading frames (98%) and 5′-untranslated regions (5′-UTRs). The 24% dissimilarity between the two cDNAs is contributed by nucleotide differences and deletions, and the major difference between them is contributed by the sequences in the 3′-UTRs.

Alignment of the amino acid sequences of GhUBC1 and GhUBC2 shows 98% identity with only three amino acid differences. Leucine (L), proline (P), and cysteine (C) are present in GhUBC1 at amino acid positions 37, 121, and 139, respectively, whereas isoleucine (I), leucine (L), and arginine (R) are present in GhUBC2 at these corresponding locations. The difference at amino acid 139 is considered as a non-conservative change. Comparison of GhUBC1 and GhUBC2 with other class I E2s that are highly similar to yeast ScUBC4/5 (Fig. 2a) revealed that GhUBC1–2 are 94–96% identical to dicotyledonous plant E2s of *A. thaliana* AtUBC8–10, tomato LeUBC, and pea PsUBC4; 89–90% identical to monocotyledonous plant E2 of rice OsUBC; 76–80% identical to human HsUbcH5B and HsUbcH5C, rat RnUBC4-1 and RnUBC4-testis, nematode CeUBC-2, fruit fly DmUbcD1, fungus CgUBC1, and yeast ScUBC4 and ScUBC5.

3.2. Southern blot analysis and genomic origins of GhUBC1 and GhUBC2

Using a *cUbc23* DNA fragment, corresponding to 1–251 bp of *GhUBC2* cDNA, as a probe for Southern blot analysis, two hybridized DNA bands were detected in the *G. hirsutum* genomic DNA digested with restriction enzymes *Dra*I, *Eco*RI, *Hin*cII, *Hin*dIII, and *Xba*I, respectively (data not shown). These results indicate that *GhUBC1* and *GhUBC2* represent two members of a subfamily of *ScUBC4/5*-related

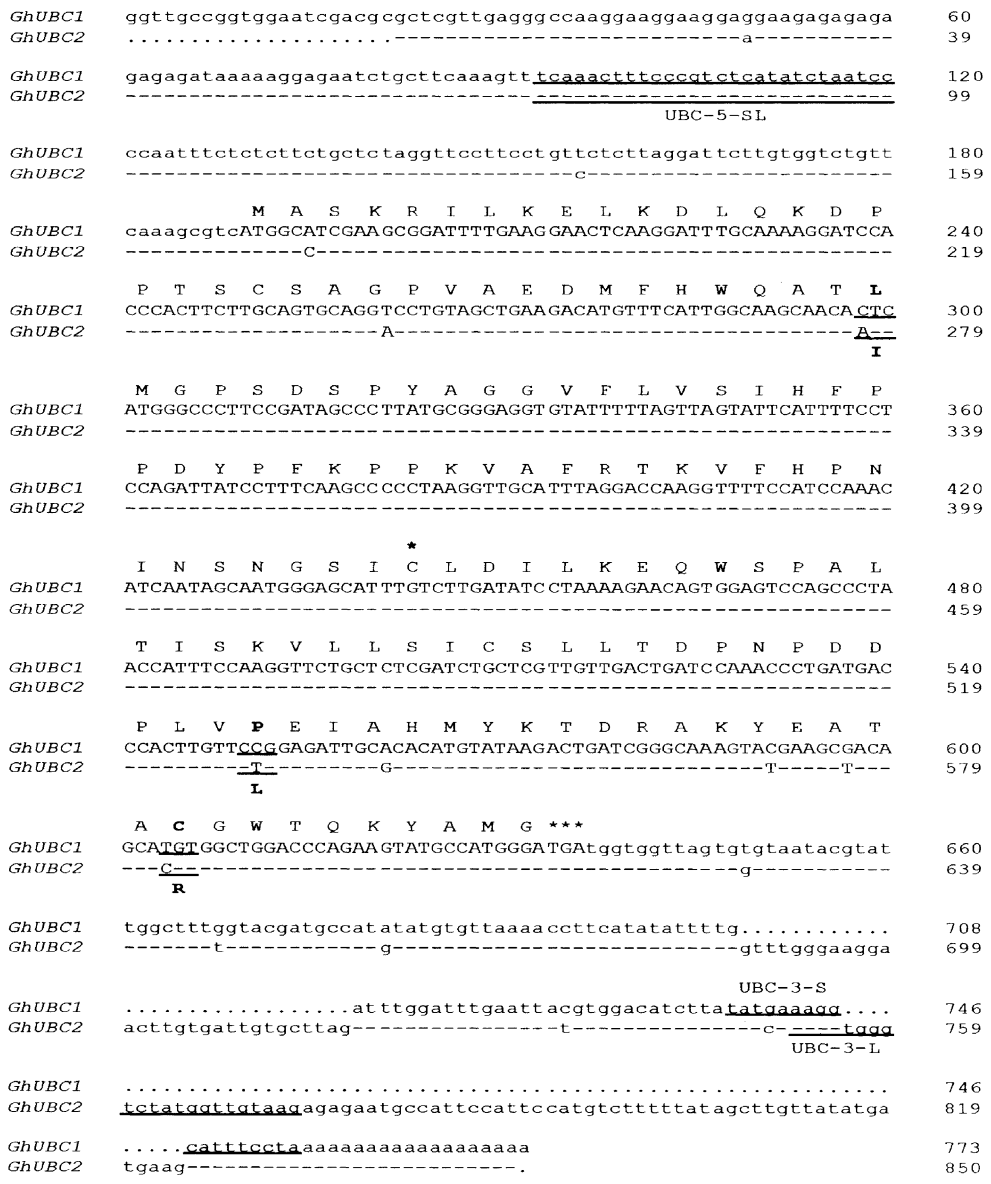


Fig. 1. Comparison of nucleotide and derived amino acid sequences of *GhUBC1* and *GhUBC2* cDNAs encoding E2 enzymes from *G. hirsutum*. Dash lines indicate identical nucleotide sequences, and the gap marked with dot lines is introduced to maximize the alignment of the two cDNAs. Three codons encoding different amino acids between GhUBC1 and GhUBC2 proteins are underlined, and their encoded amino acids are in bolded faces. Three asterisks are used to represent the stop codon. The active site cysteine required for the thioester bond formation with ubiquitin is indicated by an asterisk. Nucleotide sequences in the 5′ and 3′ untranslated regions are shown in lower cases, and those in the ORF regions are represented in upper cases. The PCR primers used in the determination of genomic origins are underlined.

E2 genes, potentially containing as many as two individuals. It is likely that all individual members in the *GhUBC1/2* gene family have been cloned. To confirm that *GhUBC1* and *GhUBC2* are the only members in the gene family, the conserved *GhUBC* coding region could be used as the probe for hybridization.

Fig. 2. Amino acid sequence comparison and phylogenetic analysis of the five class I E2s from *Gossypium* and their closely related E2s from other organisms. GhUBC1 and GhUBC2 (*G. hirsutum*, allotetraploid $(AD)_1$ genome); GaUBC1 (*G. arboreum*, A_2 genome); GtUBC2 (*G. thurberi*, D_1 genome); GrUBC2 (*G. raimondii*, D_5 genome); AtUBC8, AtUBC9, and AtUBC10 [13] (*A. thaliana*, accession numbers P35131, P35132, and P35133); LeUBC [14] (*L. esculentum*, accession number X73419); PsUBC4 [15] (*P. sativum*, accession number AAA64427); OsUBC [16] (*O. sativa*, accession number S61417); HsUbcH5B and HsUbcH5C [12] (*H. sapiens*, accession numbers AAA91460 and AAA91461); RnUBC4-1 and RnUBC4-testis [9–11] (*R. norvegicus*, accession numbers AAA85102 and AAC52942); CeUBC-2, [8] (*C. elegans*, accession number AAB25489); DmUbcD1 [6] (*D. melanogaster*; accession number P25867); CgUBC1 [7] (*C. gloeosporioides*, accession number AAC39499); ScUBC4 and ScUBC5 [5] (*S. cerevisiae*, accession numbers P15731 and P15732). (a) Identical amino acids are indicated by dashed lines. The cysteine residue at the active site is marked with an asterisk. The eight amino acids that are conserved in human, animals, fungus, and yeast but not in plants are boxed. The eleven amino acid residues conserved in plants but not in other organisms are indicated by open arrowheads. (b) A phylogenetic tree based on amino acid sequences of the five cotton E2s and their closely related E2s from other organisms.

The genomic origins of *GhUBC1* and *GhUBC2* genes were determined by PCR using two sets of primers, UBC-3-S/UBC-5-SL and UBC-3-L/UBC-5-SL (Fig. 1), in the amplification of genomic DNAs isolated from *G. herbaceum* (A_1), *G. arboreum* (A_2), *G. thurberi* (D_1), *G. raimondii* (D_5), and *G. hirsutum* St213 ($(AD)_1$), respectively. The

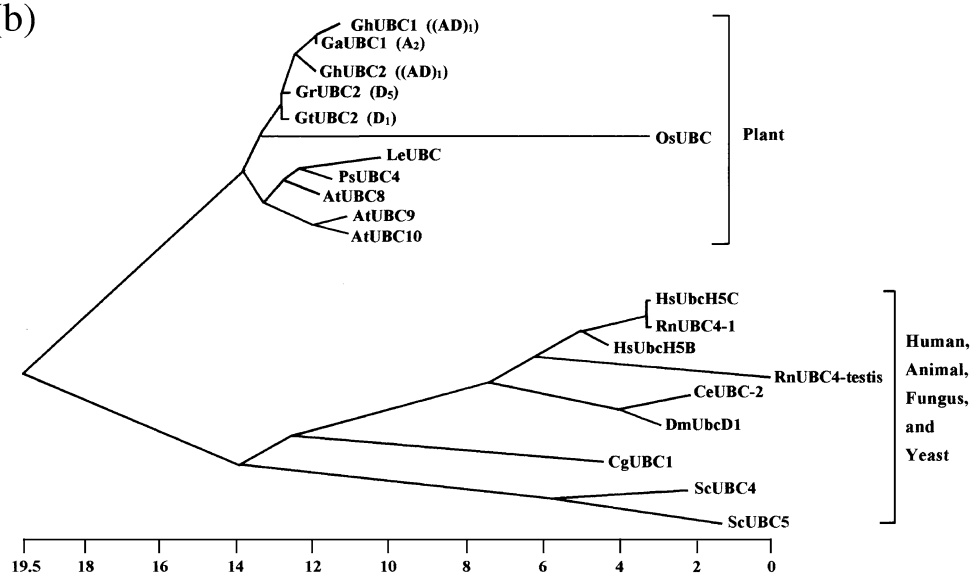
(a)

```
                    10        20        30        40        50
GhUBC1      MASKRILKEL KDLQKDPPTS CSAGPVAEDM FHWQATLMGP SDSPYAGGVF
GaUBC1      ---------- ---------- ---------- ---------- ----------
GhUBC2      ---------- ---------- ---------- ------I--- ----------
GtUBC2      ---------- ---------- ---------- ------I--- ----------
GrUBC2      ---------- ---------- ---------- ------I--- ----------
AtUBC8      ---------- ---------- ---------- ------I--- AE-S------
AtUBC9      ---------- ---------- ---------- ------I--- -------S--
AtUBC10     ---------- ---------- ---------- ------I--- -E--------
LeUBC       ---------- ---------- ---------- ------I--- A-----S---
PsUBC4      ---------- ---------- ---------- ------IV-- A---------
OsUBC       --------FC- -CTP------ ---------- ------I--- P---------
HsUbcH5B    --L---H--- N--AR---AQ ------GD-- ------I--- N----Q----
HsUbcH5C    --L---N--- S--AR---AQ ------GD-- ------I--- N----Q----
RnUBC4-1    --L---N--- S--AR---AQ ------GD-- ------I--- N----Q----
RnUBC4-T    --L---H--- N--AQ---AQ ------G--- ------I--- N----Q--A-
CeUBC-2     --L---Q--- Q--GR---AQ ------GD-L ------I--- PE---Q----
DmUbcD1     --L---N--- Q--GR---AQ ------GD-L ------I--- P----Q----
CgUBC1      --F---N--- T--GR---S- ------G--L ------I--- G----S----
ScUBC4      MSS----A--- S--ER----- ------GD-L Y----SI--- A---------
ScUBC5      MSS----A--- S--GR---A- ------GD-L Y----SI--- ----------

                    60        70        80        90       100
GhUBC1      LVSIHFPPDY PFKPPKVAFR TKVFHPNINS NGSICLDILK EQWSPALTIS
GaUBC1      ---------- ---------- ---------- ---------- ----------
GhUBC2      ---------- ---------- ---------- ---------- ----------
GtUBC2      ---------- ---------- ---------- ---------- ----------
GrUBC2      ---------- ---------- ---------- ---------- ----------
AtUBC8      --T------- ---------- ---------- ---------- ----------
AtUBC9      --T------- ---------- ---------- ---------- ----------
AtUBC10     --T------- ---------- ---------- ---------- ----------
LeUBC       --T------- ---------- ---------- ---------- ----------
PsUBC4      --T------- ---------- ---------- ---------- ----------
OsUBC       ---------- --NR-----K ---------- ---------- ---------V-
HsUbcH5B    FLT----T-- ---------T -RIY------ ---------R S---------
HsUbcH5C    FLT----T-- ---------T -RIY------ ---------R S---------
RnUBC4-1    FLT----T-- ---------T -RIY------ ---------R S---------
RnUBC4-T    FLT-D--TE- -------ET -RIY---V-- ---------R S---------
CeUBC-2     FLT----T-- ---------T -RIY------ ---------R S---------
DmUbcD1     FLT----T-- ---------T -RIY------ ---------R S---------
CgUBC1      FLA----T-- -------NT -RIY------ ---------R D---------
ScUBC4      FL-------- ------IST --IY-----A --N------- D------L--
ScUBC5      FL-----T-- -------NT --IY------ S-N------- D------L--

                   110       120       130       140       148
GhUBC1      KVLLSICSLL TDPNPDDPLV PEIAHMYKTD RAKYEATACG WTQKYAMG
GaUBC1      ---------- ---------- ---------- --------R- --------
GhUBC2      ---------- ---------- L--------- --------R- --------
GtUBC2      ---------- ---------- ---------- --------R- --------
GrUBC2      ---------- ---------- ---------- --------R- --------
AtUBC8      ---------- ---------- ---------- --------RN --------
AtUBC9      ---------- ---------- ---------- KN---S--RT --------
AtUBC10     ---------- ---------- ---------- KN---S--RS --------
LeUBC       ---------- ---------- ---------- -S---T--RS ----F---
PsUBC4      ---------- ---------- ---------- -T------RS --------
OsUBC       ----------V---------- --M------- -----S--RG --------
HsUbcH5B    ---------- C--------- -----RI---- -E--NRI-RE --------
HsUbcH5C    ---------- C--------- -----RI---- -D--NRISRE --------
RnUBC4-1    ---------- C--------- -----RI---- -D--NRISRE --------
RnUBC4-T    -------S-- C--------- -----QI---- -D--NR--RE --------
CeUBC-2     ---------- C--------- -----RI---- -ER-NQL-RE --------
DmUbcD1     ---------- C--------- -----RI---- -E--NEL-RE --R-----
CgUBC1      --------M- -------E-- -----V----- --R-----RE --R----I
ScUBC4      ---------- --A------- -----I----- -P------RE --K---V
ScUBC5      ---------- --A------- -----QI---- K--------E --K---V
```

(b)



UBC-3-S and UBC-3-L primers were specifically designed from the 3′ end of the *GhUBC1* and *GhUBC2* cDNA sequences, respectively, so that only the corresponding genomic DNA sequences could be amplified. A 2-kb DNA fragment was amplified only from A_2 and $(AD)_1$ genomes with the primers UBC-5-SL and UBC-3-S (data not shown), suggesting that *GhUBC1* is present in the A subgenomes of *G. hirsutum* and the diploid cotton *G. arboreum* (A_2) is more likely to be the ancestral *GhUBC1* gene donor. The other set of primers, UBC-3-L and UBC-5-SL, could only amplify the 2-kb fragment from D_1, D_5, and $(AD)_1$ genomes, indicating that *GhUBC2* is present in the D

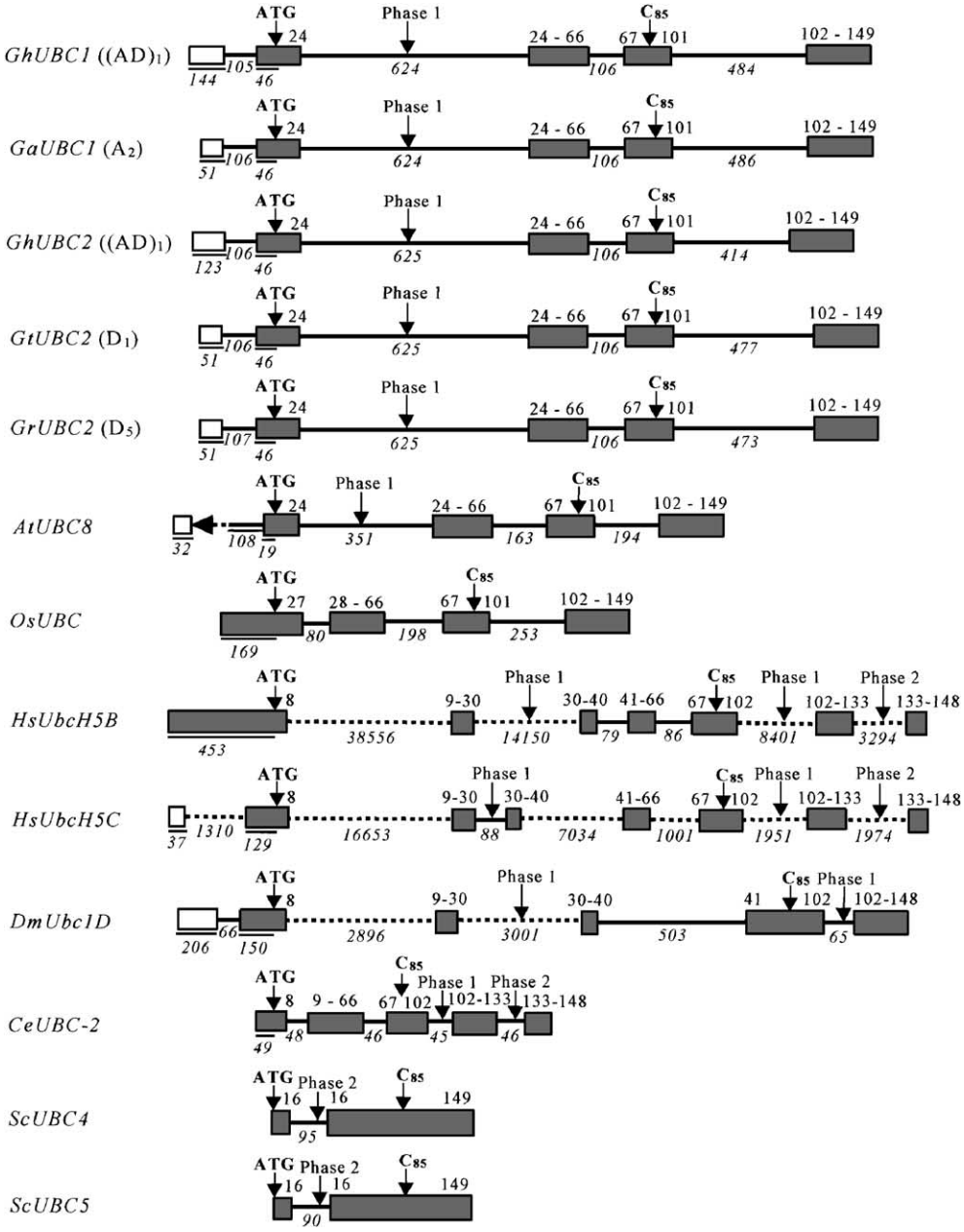


Fig. 3. Diagrammatic comparisons of genomic structures of yeast *ScUBC4/5* and their homologs from different organisms. The thirteen E2 genes include *GhUBC1* and *GhUBC2* from *G. hirsutum*, *GaUBC1* from *G. arboreum*, *GtUBC2* from *G. thurberi*, *GrUBC2* from *G. raimondii*, *AtUBC8* from *A. thaliana*, *OsUBC* from *O. sativa*, *HsUbcH5B* and *HsUbcH5C* from *H. sapiens*, *CeUBC-2* from *C. elegans*, *DmUbcD1* from *D. melanogaster*, *ScUBC4* and *ScUBC5* from *S. cerevisiae*. The E2 genes are aligned with respect to the position of the start codon ATG. The codon 85 is indicated by C_{85}, which encodes the active site cysteine. The exons containing the coding regions are represented by filled boxes, whereas the exons located in the 5′ untranslated region are indicated by open boxes. The positions of codons at the 5′ and 3′ ends within exons are indicated. The codon numbers 148 and 149 represents the position of the stop codons. The sizes (base pairs) of introns and 5′ -untranslated regions present in the mRNA are marked in italic under the genes. The lengths of exons and introns are represented in their relative sizes except for the large introns in *HsUbcH5B*, *HsUbcH5C*, and *DmUbc1D*, where the introns are indicated with broken lines. Arrows mark the introns in either phase 1 or 2 position. The phase 1 or 2 intron interrupts the first and the second or the second and the third nucleotides in the same codon, and is marked by phase 1 or phase 2, respectively.

subgenomes of *G. hirsutum*, and *G. thurberi* (D_1) or *G. raimondii* (D_5) is possibly the ancestral *GhUBC2* gene donor. The data, however, are unable to distinguish whether *G. thurberi* (D_1) or *G. raimondii* (D_5) is more closely related to the ancestral *GhUBC2* gene donor.

3.3. Determination and comparison of the genomic sequences of GhUBC1/2 and their homologous genes GaUBC1, GtUBC2, and GrUBC2 from the diploid A_2, D_1, and D_5 genome cottons

The five 2-kb DNA fragments, representing the E2 genomic sequences amplified from tetraploid and diploid genomes, were purified from the agarose gel, cloned into pGEM-T Easy vector (Promega), and sequenced by vector primers (T7 and Sp6) and the internal primers designed from the identified sequences. In accordance with the nomenclature of *GhUBC1* and *GhUBC2*, the genomic sequences amplified from *G. arboreum* (A_2), *G. thurberi* (D_1), and *G. raimondii* (D_5) were designated as *GaUBC1*, *GtUBC2*, and *GrUBC2*, respectively.

The MegAlign program (DNASTAR) was used to align the genomic sequences of *GhUBC1* and *GaUBC1* with the cDNA sequence of *GhUBC1*, and the genomic sequences of *GhUBC2*, *GrUBC2*, and *GtUBC2* with the cDNA sequence of *GhUBC2* by the Clustal method. The alignment revealed that the five cotton *ScUBC4/5*-related E2 genes contained five exons and four introns with three introns located within the ORF and one intron at the 5′-UTR region (Fig. 3). All predicted intron boundaries had the invariant 5′-GT. . .AG-3′ as splicing donor and acceptor sites. Intron 1 was located at the 5′-UTR, and introns 2, 3, and 4 interrupted the coding sequences. Intron 2 is a phase 1 intron [24], which interrupts a codon between the first and second base positions. Introns 3 and 4 are phase 0 introns [24], which interrupt the coding sequence between adjacent codons.

The genomic sequences in the exon regions of *GhUBC1* and *GhUBC2* are completely identical with their corresponding cDNA sequences. The deduced amino acid sequences encoded by the predicted exons of *GaUBC1*, *GtUBC2*, and *GrUBC2* genes from diploid A_2, D_1, and D_5 genome cottons were aligned with those of GhUBC1, GhUBC2, and the ScUBC4/5-related E2s from other organisms (Fig. 2a). A phylogenetic tree was constructed from the comparison of the amino acid sequences of the E2s by the Clustal method (Fig. 2b). Since GtUBC2 (D_1) and GrUBC2 (D_5) proteins have the same amino acid sequences, the phylogenetic tree could not determine which protein had a closer relationship with the GhUBC2 protein.

The Clustal method was also used to compare the exon and intron sequences of *GhUBC1*, *GaUBC1*, *GhUBC2*, *GtUBC2*, and *GrUBC2*, and a phylogenetic tree was drawn to estimate the evolutionary relationship among the five cotton E2 genes. The results (data not shown) confirmed that *G. arboreum* (A_2 genome) is more likely to be the inherited donor of *GhUBC1*, and *G. raimondii* (D_5 genome) but not *G. thurberi* (D_1 genome) is more closely related to the hereditary donor of *GhUBC2*. The comparison of the five *Gossypium* E2 genes revealed a high percent nucleotide identity among them: 95.9% identity between *GhUBC1* and *GhUBC2*, 99.7% between *GhUBC1* and *GaUBC1* (A_2), 98.6% between *GhUBC2* and *GtUBC2* (D_1), and 98.7% between *GhUBC2* and *GrUBC2* (D_5).

3.4. Functional complementation of yeast ScUBC4/5 by cotton GhUBC1 and 2

A yeast complementation experiment was conducted to determine whether *GhUBC1* and *GhUBC2* have the same functions as *ScUBC4* and *ScUBC5*. The deletion of both *ScUBC4* and *ScUBC5* drastically impaired growth in yeast

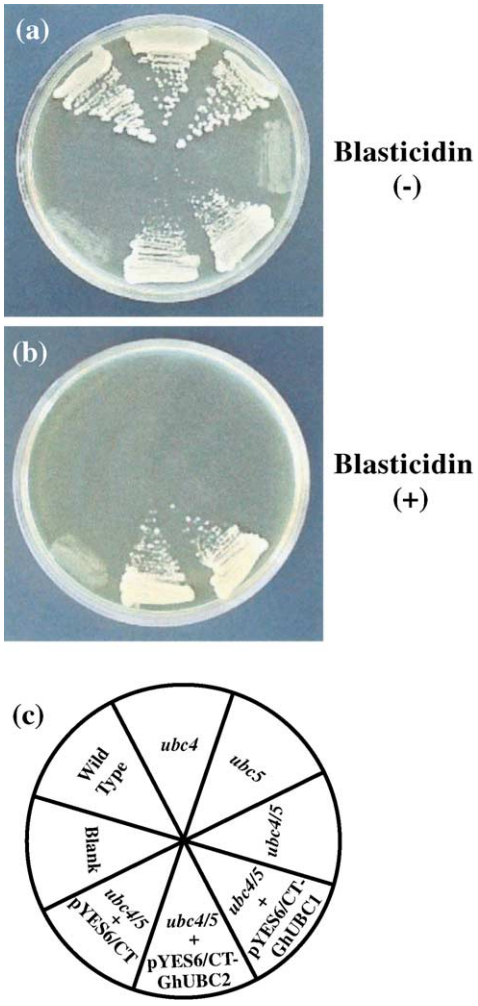


Fig. 4. Functional complementations of yeast ScUBC4/5 by cotton GhUBC1 and GhUBC2. The yeast *ubc4ubc5* (*ubc4/5*) double mutant was individually transformed with pYES6/CT-GhUBC1, pYES6/CT-GhUBC2, and pYES6/CT vectors, and selected on Blasticidin-YPG medium plates. The wild type, *ubc4*, *ubc5*, *ubc4/5*, along with three *ubc4/5* transformants harboring pYES6/CT, pYES6/CT-GhUBC1, and pYES6/CT-GhUBC2, respectively, were streaked on YPG plates with (b) and without (a) Blasticidin. The location of yeast strains streaked on the YPG plates is shown in the diagram (c).

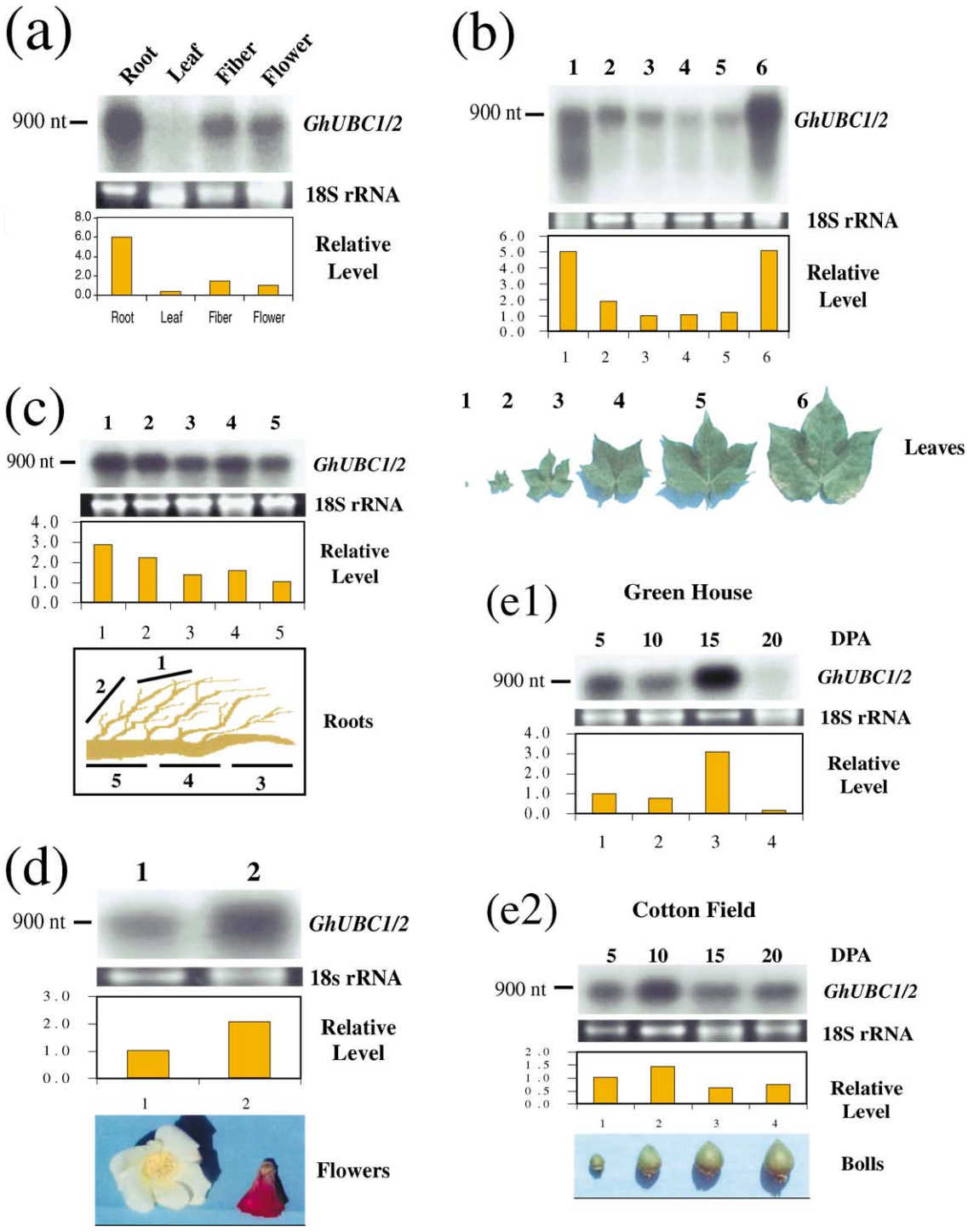


Fig. 5. Northern blot analysis of *GhUBC1/2* expression. RNA samples are from: (a) whole roots, fully expanded young leaves, 15-DPA fibers (from green house), and cream flowers; (b) leaves in a main branch at different developmental stages (lane 1: the first folded young leaf, lane 2: the first open young leaf, lane 3: the second fully expanded young leaf, lane 4: the third matured leaf, lane 5: the fourth matured leaf, and lane 6: the fifth senescent leaf.); (c) the apical (lane 1) and matured parts (lane 2) of lateral roots, and the apical (lane 3), middle (lane 4), and matured (lane 5) parts of primary roots (Note: The apical part of primary root doesn't contain the root-tip/meristem/elongation zones which were lost during sample collection); (d) flowers at two different developmental stages (lane 1: opened flowers in cream color, and lane 2: withered flowers in pink color); (e) fibers at different developmental stages (5, 10, 15, and 20 DPA) grown in a green house (e1) and the field (e2). The relative *GhUBC1/2* transcript levels were determined by the ratio of hybridized intensity of the 0.9-kb E2 mRNA to the EtBr-stained 18S rRNA band using the program of Scion Image for Windows (Scion Corporation, http://www.scioncorp.com).

ubc4ubc5 cells, whereas the growth rate of *ubc4* and *ubc5* single mutants was almost similar to the wild-type yeast. The growth doubling times for wild-type *ubc4*, *ubc5*, and *ubc4ubc5* yeast cells in YPD liquid medium at 30 °C are 1.5, 2.0, 1.5, and 16 h, respectively [5]. Because of the structural similarity between ScUBC4 and ScUBC5 proteins, *ScUBC4* and *ScUBC5* genes can complement with each other in their function [5].

The yeast *ubc4ubc5* double mutant was transformed with a recombinant plasmid pYES6/CT-GhUBC1 or pYES6/CT-GhUBC2, and the transformants were selected on YPD plates containing the antibiotic of blasticidin. The transformed *ubc4ubc5* mutant, harboring either GhUBC1 or GhUBC2, grew much faster than the untransformed mutant or the mutant transformed with the pYES6/CT vector, and formed colonies with a similar size as wild-type *ubc4* and *ubc5* single mutants on YPG plates incubated at 30 °C (Fig. 4). These data suggested that cotton *GhUBC1* and *GhUBC2* genes have similar functions as the yeast *ScUBC4* and *ScUBC5* genes, which are involved in the selective degradation of abnormal and short-lived proteins.

3.5. Spatial and temporal expression of GhUBC1/2

Northern blot analysis with the *GhUBC2* cDNA probe showed that a 0.9-kb mRNA was expressed in leaves, fibers, flowers, and roots in a developmentally regulated manner (Fig. 5a–e). Because of the high similarity between the cDNA sequences of *GhUBC1* and *2*, the cDNA probe could not distinguish the expression between the *GhUBC1* and *GhUBC2* genes. Semi-quantitative RT-PCR [25,26] with the two sets of gene-specific primers (GhUBC-5-SL/GhUBC-3-S and GhUBC-5-SL/GhUBC-3-L) demonstrated that *GhUBC1* and *GhUBC2* shared similar transcriptional patterns as in Northern blot analysis (data not shown). The highest level of *GhUBC1/2* expression was detected in roots, while a low level of expression was observed in the fully expanded young leaves (Fig. 5a). *GhUBC1/2* were expressed at all stages of leaf development with a significantly increased expression in the first folded young leaf (the youngest leaf) and the fifth senescent leaf (the oldest leaf) (Fig. 5b). The *GhUBC1/2* transcripts were present in all parts of roots but with the highest levels in the apical part of lateral roots (Fig. 5c). The expression of *GhUBC1/2* is higher in senescent pink-colored flowers than cream-colored flowers in full bloom (Fig. 5d). The transcript level of *GhUBC1/2* in fibers reached a maximum at 15 DPA when cotton was grown in the green house (Fig. 5e1). The highest expression of *GhUBC1/2* in the fibers collected from the field, however, was at 10 DPA (Fig. 5e2). Cotton bolls in the field generally developed faster and had larger sizes than those in the green house (data not shown). Because the 10 DPA bolls from the field had similar sizes as 15 DPA bolls from the green house, the 10 DPA and 15 DPA fibers were considered to be at similar development stages.

4. Discussion

The seven plant E2 genes, including cotton *GhUBC1*, *GaUBC1*, *GhUBC2*, *GtUBC2*, and *GrUBC2*, *Arabidopsis AtUBC8*, and rice *OsUBC*, all contain three introns within their ORFs, but the five cotton genes and *Arabidopsis AtUBC8* have an extra intron in their 5′ -UTRs (Fig. 3). Although the seven plant genes encode the same size proteins (148 amino acids), the sizes of their introns are quite different. Besides having the same numbers of introns, the structural similarity among the five cotton E2 genes and *AtUBC8* is also indicated by the locations of their introns. The positions of the second and third introns among the seven plant E2 genes are identical and are located between codons 66 and 77 and between codons 101 and 102, respectively. The first introns within the ORF regions among the five *Gossypium* E2 genes and *AtUBC8* are phase 1 introns and interrupt codon 24 between the first and the second nucleotides, whereas the first intron in *OsUBC* is a phase 0 intron and located between codons 27 and 28. Since both cotton and *Arabidopsis* are dicotyledonous plants whereas rice is a monocotyledon, the structures of the five *Gossypium* E2 genes are therefore more closely related with *Arabidopsis AtUBC8* than with rice *OsUBC*. The exon and intron structures of plant E2 genes are more complicated than those of *ScUBC4* and *ScUBC5* from the yeast. Both yeast *ScUBC4* and *5* have only one phase 2 intron, which interrupts codon 16. The intron positions within the ORFs in animals are also highly conserved but entirely different from those in the plant. The human class I E2 genes, *HsUbcH5B* and *HsUbcH5C*, contain six introns, and their intron positions are identical between the two genes. However, the intron sizes between the two human genes vary largely. Although the fruit fly *DmUbc1D* and nematode *CeUBC-2* genes have fewer introns than those from human, the positions of the two introns, which interrupt codons 8 and 9 (phase 0) and codon 102 (phase 1), respectively, are identical with those in human *HsUbcH5B* and *HsUbcH5C*. It appears that as organisms become more evolved, more introns are found within their class 1 E2 genes, and in general the sizes of their introns also increase.

G. hirsutum, known as upland cotton, is an allotetraploid $(AD)_1$ genome species that evolved in the New World. It contains one subgenome that originated from the Old World diploid A-genome cotton and another subgenome from the New World diploid D-genome cotton. Among the 13 New World D-genome diploid species, *G. raimondii* (D_5 genome) is considered as the likely D genome donor to *G. hirsutum* ($(AD)_1$ genome), whereas *G. herbaceum* (A_1 genome) is suggested to be the A genome donor from the only two known Old World A-genome diploid species, *G. herbaceum* and *G. arboreum* (A_2 genome) [27,28]. Our genomic origin analysis indicates that *GhUBC1* is present in the A subgenome and *GhUBC2* in the D subgenome of *G. hirsutum*, and suggests that *G. raimondii* (D_5) is the D genome donor and *G. arboreum* (A_2) is likely the A genome donor of *G.*

hirsutum. Two acetohydroxyacid synthase (AHAS) genes were identified in *G. hirsutum* and assigned to A and D subgenomes, respectively [29]. The *G. hirsutum* tonoplast intrinsic proteins (TIPs) were encoded by two large gene subfamilies; one subfamily is assigned to A subgenome, whereas the other is assigned to D subgenome [30]. Phylogenetic analysis of alcohol dehydrogenase (*Adh*) gene family in *G. hirsutum* indicates that *AdhA*, *AdhB*, *AdhC*, *AdhD*, and *AdhE* genes located in the D-subgenome of *G. hirsutum* likely originated from the D-genome diploid *G. raimondii*, and *AdhA*, *AdhB*, *AdhD*, and *AdhE* genes in the A-subgenome of *G. hirsutum* probably originated from the A-genome diploid *G. herbaceum* [28]. The *AdhC* gene is present in *G. arboretum* but is missing from *G. herbaceum*, suggesting that *G. arboretum* is likely the ancient donor of *AdhC* present in the A-subgenome of *G. hirsutum*. Similarly, *GaUBC1* can only be amplified from *G. arboretum* among the diploid cottons. Although *G. herbaceum* has long been suggested to be the ancient donor of the A-subgenome in *G. hirsutum*, the presence of *AdhC* and *GaUBC1* genes only in *G. arboretum* but their absence in *G. herbaceum* implicate that *G. arboretum* could be also the A-subgenome donor of *G. hirsutum*. However, it is unlikely that *GhUBC1* is absent in *G. herbaceum* since it is present as a single-copy housekeeping gene in the diploid cotton.

ScUBC4/5 and their homologs are members of class I E2s and require supplementary E3s for substrate recognition [4]. It has been reported that the class I E2s from nematode, fruit fly, and fungus can substitute the function of ScUBC4/5 in yeast cells [6–8]. Here, we have shown for the first time that plant class I E2s, GhUBC1/2, are also able to complement the function of ScUBC4/5. Comparison of the E2 protein sequences (Fig. 2a) among plants, human, animals, fungus, and yeast has revealed that eight conserved amino acid residues including Gly^{27}, Phe^{51}, Leu^{52}, Thr^{70}, Ile^{73}, Tyr^{74}, Ile^{126}, and Glu^{140} are present in human, animals, fungus, and yeast but absent in the plants. This observation indicates that the eight conserved residues are not crucial for the basic function of ScUBC4/5 and their homologs from other organisms in yeast cells. Plant E2s, however, contain 11 conserved amino acid residues, Leu^{7}, Lys^{11}, Lys^{15}, Ala^{27}, Leu^{51}, Val^{52}, Arg^{70}, Val^{73}, Phe^{74}, Glu^{91}, and Met^{126}, that are absent in other organisms (Fig. 2a). Oughtred et al. [9] reported that site-directed mutagenesis of four amino acid residues away from the active-site cysteine of a rat ScUBC4/5 homolog, RnUBC4-1, can alter its interaction with E3 or substrate and result in the conjugation of ubiquitin to different target proteins. Phylogenetic analyses indicated that the plant homologs are more closely related among themselves but distant from the cluster of yeast ScUBC4/5 and their homologs from human, animal, and fungus (Fig. 2b). The distinct primary structure of the 11 conserved amino acid residues in the plant E2s (Fig. 2a) may have an important role in substrate specificities for targeting ubiquitin to plant-specific proteins, such as the repressor-like protein in rice aleurone cells during GA response [16].

Many studies suggest that the ubiquitin-proteasome pathway is involved in the plant senescence program. The tomato *LeUBC* [14] and *Arabidopsis AtUbc4a* and *AtUbc4b* [31] were observed with increased expression during leaf senescence. The levels of ubiquitin mRNAs and *GUS* expression driven by an ubiquitin promoter also increased during leaf senescence [31,32]. In this study, elevated levels of cotton *GhUBC1/2* mRNAs were observed in both senescent leaves and flowers. An F-box protein, ORE9, has been suggested to regulate leaf senescence in *Arabidopsis* via the ubiquitin-proteasome pathway [33]. An SCF complex, SCF^{ORE9}, containing the ORE9 protein can promote leaf senescence by selective degradation of target proteins that are essential for the delay of the leaf senescence program. During plant senescence, various hydrolytic activities are involved in the massive disintegration of cells, and the degraded cellular materials including many macromolecules are then redistributed as nutrients to other parts of plant [34]. Many proteolytic enzymes, such as cysteine proteases and aspartic proteases, are involved in protein turnover. The study of the function of ORE9 has suggested that the ubiquitin-proteasome pathway may not be involved in the massive turnover of proteins during senescence but instead mediate the degradation of specific proteins [33]. The increased expression of GhUBC1/2 in senescent tissues suggests that these E2s may interact with the E3 complex, SCF^{ORE9}, in conjugating ubiquitin to the specific target proteins for degradation. Since the delay of senescence and prolonged flower-set period for several weeks could significantly increase the yield of cotton fiber from 10% to 30% [35], the better understanding of the senescence program regulated by ubiquitin-dependent pathway in cotton would lead to the development of the genetically engineered cotton with higher fiber production.

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